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Sex-specific effects of maternal testosterone on lateralization in a cichlid fish

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Lateralization of cerebral functions is a fundamental aspect of the organization of brain and behaviour in vertebrates. Sex differences in human lateralization have inspired researchers to postulate several hypotheses concerning the effect of prenatal testosterone on lateralization, but few experimental studies have examined these hypotheses. We investigated whether prenatal testosterone affects strength or direction of lateralization in a cichlid fish, *Aequidens rivulatus*. Eggs were given a control or testosterone treatment immediately after spawning, mimicking elevated maternal androgen concentrations towards the high end of the natural range. After 7 months the fish were tested in two rotational preference tests. As expected from earlier studies, control fish showed (nonsignificant) right-eye preference while viewing a predator and (significant) left-eye preference while viewing their mirror image, but no clear sex differences were apparent. A sex-specific effect of our treatment was found in the first test. Only females exposed to elevated prenatal levels of testosterone significantly shifted in direction of lateralization. In the second test no effect of the treatment was found. Our results suggest that mothers have a stronger influence on the lateralization pattern of their daughters than on their sons, but do not support any of the current hypotheses about prenatal testosterone and development of lateralization.
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Lateralization, the asymmetric specialization of functions in the brain, is apparent in most, if not all, vertebrate animals (Vallortigara & Bisazza 2002; MacNeilage et al. 2009; Vallortigara et al. 2011). In humans, sex differences in lateralization (for a meta-analysis see Papadatou-Pastou et al. 2008) has inspired researchers to hypothesize that prenatal testosterone (pT) is involved in the development of lateralization (reviewed in Pfannkuche et al. 2009). One well-cited hypothesis was put forward by Geschwind & Galaburda (1985) and proposed that pT slows neuronal growth in the left hemisphere and consequently leads to compensatory growth in the right hemisphere. Increased levels of pT would thus lead to increased dominance of the right hemisphere. This hypothesis has been extensively criticized in the literature (e.g. Bryden et al. 1994; Berenbaum & Denburg 1995). Another hypothesis, the sexual differentiation hypothesis, proposes that lateralization is related to the process of sexual differentiation and that early exposure to testosterone causes masculinization of lateralization (Hines & Shipley 1984). Finally, the callosal hypothesis states that pT increases axonal pruning in the corpus callosum of males, which leads to a decrease in communication between the hemispheres and therefore increases the strength of lateralization (Witelson & Nowakowski 1991). This hypothesis only applies to mammals, as nonmammals have no anatomic structure homologous to the corpus callosum.

Although these hypotheses were inspired by sex differences in human lateralization, testing them on humans is limited by ethical concerns and human research has therefore focused on an observational approach. Unfortunately this approach, using either nonrandom clinical samples of individuals with abnormal prenatal exposure to gonadal hormones or indirect markers for pT, has lead to ambiguous results. Some studies, using clinical samples, found that girls prenatally exposed to higher levels of testosterone show enhanced left-hemisphere dominance of language (Hines & Shipley 1984), whereas others found no effect on language lateralization (Smith & Hines 2000; Mathews et al. 2004). Similar studies found that high pT leads to reduced right-hand preference (Schachter 1994; Mathews et al. 2004), others to increased left-handedness (Nass et al. 1987; Scheirs & Vingerhoets 1995; Kelso et al. 2000; Smith & Hines 2000) and one study found no effect on handedness (Helleday et al. 1994). Furthermore, some studies, using indirect markers for pT such as 2D:4D digit ratio, adult testosterone levels or sex of the co-twin, found a positive correlation between high levels of pT and right-handedness (Moffat & Hampson 1996; Gadea 2003), whereas others found a negative (Tan 1991) or no correlation (Beaton et al. 2011). Only three studies measured pT directly in amniotic fluid and correlated this with lateralization patterns of the resulting children. Again inconsistent results were found: Grimshaw et al. (1995) found that higher pT exposure was related to increased left-hemisphere specialization of speech in

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girls and not in boys whereas Lust et al. (2010) found a stronger effect in boys, although this did not result in a significant sex effect. Furthermore, the first study found that higher pT was associated with increased right-handedness in girls, but not in boys (Grimshaw et al. 1995), whereas another study found no such correlation between pT and handedness (Lust et al. 2011). Few studies have taken an experimental approach in investigating the effects of pT exposure on lateralization in nonhuman animals and, in accordance with the observational studies in humans mentioned above, these studies often found sex-specific effects. Testosterone treatment administered on day 16 of incubation in chickens, *Gallus gallus domesticus*, reversed the pattern of asymmetry in the brain of males and decreased lateralization in females (Schwarz & Rogers 1992). Prenatal testosterone treatment also moderately shifted the population-level lateralization of tail posture in female Wistar rats, *Rattus norvegicus*, whereas in males there was no effect (Rosen et al. 1983). These studies highlight the importance of studying lateralization in general, and the effect of testosterone on lateralization in particular, with respect to the sex of the animal. A meta-analysis showed that the data available thus far, on both human and nonhuman animals, do not support any of the three hypotheses mentioned above, possibly because of the small number of experimental studies investigating the effects of pT on lateralization (Pfannkuche et al. 2009).

To investigate experimentally the effect of pT on lateralization and its sex specificity, we used the cichlid fish species *Aequidens rivulatus* as our model organism. Cichlid fish are oviparous, facilitating easy manipulation of pT levels in the eggs outside the mother's body. The pT can originate from both the embryo itself and from its mother. Congruent with avian species (reviewed in von Engelhardt & Groothuis 2011), egg yolk of fish contains substantial levels of maternal steroid hormones, including androgens (Schreck et al. 1991; McCormick 1998; Eriksen et al. 2006) that can affect offspring development in several fish species (McCormick 1999; Gagliano & McCormick 2009; Sloman 2010). Since maternal hormone production is under the influence of the environment, a mother can communicate the prevailing conditions to her offspring by means of depositing different amounts of gonadal hormones into her eggs, influencing physiology, morphology and behaviour, often in a sex-specific manner (birds: reviewed in Groothuis et al. 2005; Gil 2008; von Engelhardt & Groothuis 2011). In the current study we tested whether elevation of testosterone concentrations in the eggs immediately after spawning, mimicking elevated deposition of maternal androgens, affects lateralization of cichlid fish species sex specifically. Determination of lateralization is very easy in teleost fish, as they have laterally placed eyes, small overlapping visual fields, visual pathways that project almost entirely to the contralateral hemisphere and low communication between the two hemispheres (Vanegas & Ito 1983). Fish are known to use the left eye to view their mirror image (Sovrano et al. 1999, 2001; de Santi et al. 2001; Sovrano & Andrew 2006; but see Reddon & Balshine 2010) and the right eye to view a predator (Bisazza & Vallortigara 1997; Bisazza et al. 1998, 1999; Facchin et al. 1999; Brown et al. 2004). We therefore used these two stimuli to investigate the effect of our treatment on the strength and direction of lateralization.

The hypothesis postulated by Geschwind & Galaburda (1985), proposing that pT causes an increase in dominance of the right hemisphere, predicts that fish that were prenatally exposed to higher levels of testosterone will show an even stronger bias, compared to control fish, to view their mirror image with their left eye (thus increasing the strength of lateralization) as the left-eye system is controlled by the right hemisphere. Furthermore, these testosterone-treated fish would shift direction from viewing the predator with the right eye towards the left eye, which may result in a decrease in strength of lateralization. The sexual differentiation theory predicts that in pT-treated fish both sexes will show an increased masculinized lateralization pattern, as, like in mammals, testosterone influences masculinization during the process of sexual differentiation in fish (Devlin & Nagahama 2002). However, the literature on sex differences in lateralization of fish is scarce and ambiguous. Reddon & Hurd (2009) found no sex difference in the direction of lateralization in a detour test for viewing objects that had positive or negative emotional valence, but both Bisazza et al. (1998) and Sovrano et al. (1999) found a sex difference with females showing a population bias to view same-sex individuals and their mirror image with the left eye, whereas males did not show population-level lateralization, although this could be accounted for by a lack of social behaviour in males. The opposite was found by Brown et al. (2007) who found a sex difference, with males showing a population bias to look at a conspecific with the right eye whereas this was not present in females. Although these results are ambiguous they show that a sex difference in lateralization when viewing a conspecific is often found in fish. The sexual differentiation theory predicts that pT-treated females will show a more masculine pattern of lateralization than control females when viewing their mirror image. The lateralization pattern of the pT-treated females will thus resemble more the lateralization pattern of control males. As no sex effect in lateralization has been found when viewing a predator (e.g. Bisazza et al. 1998) no effect of treatment is expected in this task. As fish have no anatomic structure homologous to the corpus callosum, the callosal hypothesis will not be considered, as it cannot be tested in this species.

METHODS

Subjects, Housing and Experimental Design

Aequidens rivulatus, a sexually dimorphic, substrate-breeding cichlid fish originating from Ecuador and Peru (Stawikowski & Werner 1998) were bred in 200-litre tanks as the second generation of wild-caught fish at our laboratory (for details see Schaafsma & Groothuis 2011). These fish, like most cichlids, show elaborate schooling behaviour before reaching sexual maturity and elaborate display behaviour during social interactions. *Astronotus ocellatus*, also a South American cichlid species, was obtained from a local pet store (four individuals) and used as a predator stimulus in the rotational preference task (see below). All adult fish were held in pairs and fed dry fish flakes (TetraMin Tropical Fish Flakes) daily and additionally received frozen shrimps weekly. All tanks were connected to large biological water filtration systems in which water temperature was kept at $25 \pm 2\,^{\circ}C$. The light:dark schedule was 12:12 h.

Eight pairs of *A. rivulatus* were used to obtain eight clutches. Clutches are laid just before lights-off and spawning was checked shortly before that time. Eggs were laid on shale covered with thin polypropylene sheets. Immediately after the discovery of a clutch the shale containing the eggs was gently removed from the tank to minimize parental disturbance and immediately replaced by shale covered with clean polypropylene sheets. We then collected the eggs and started the treatment. Of these clutches half of the eggs received testosterone treatment and half a control treatment (see below). After treatment of the eggs, they were left to hatch in compartments measuring ca. 20×30 cm and 20 cm high. Fry and larvae, housed in groups split according to clutch and treatment, were fed daily with brine shrimp. We tested the fish at 7 months posthatching (standard length mean $\pm$ SD $= 3.06 \pm 0.061$ cm), before they became reproductively active. We used 92 fish, originating from four clutches, produced by four different females and

males; only four clutches were used because these were the only ones that contained sufficient fish for both control and testosterone-treated groups. The fish underwent both a rotational preference test with a live predator as stimulus (RPT-P) and a rotational preference test in which the fish could view their own mirror image (RPT-M; see below). Half of the fish first performed the RPT-P and the other half first performed the RPT-M. After the first test the fish were gently dip-netted out of the test apparatus and placed into a small opaque PVC tube (diameter 5 cm) for acclimatization in the other test apparatus for 5 min. After the last test the fish were killed for sex determination (see below).

Treatment of Eggs

The freshly laid clutches were gently taken off from the polypropylene sheet using a scalpel and divided in two, and placed in well-aerated 200 ml cups containing home tank water. One half of the clutch received the testosterone treatment, which was based on a pilot study on 11 clutches. In this pilot study we split every clutch into four and used four different dosages (0, 2, 20 or 200 μg of methyl-testosterone (MT, Sigma) dissolved in 0.1 ml of ethanol per litre of tank water) within a clutch to establish which concentration elevates the mean levels of testosterone to two times the standard deviation of control clutches.

Based on the results of this pilot study (0 μg MT: mean ± SD = 2.65 ± 2.51 μg; 2 μg MT: mean ± SD = 5.46 ± 3.92 μg; 20 μg MT: mean ± SD = 72.07 ± 56.86 μg; 200 μg MT: mean ± SD = 758.27 ± 561.10 μg) we used 3.57 μg of methyl-testosterone (Sigma) dissolved in 0.1 ml of ethanol per litre of tank water as the testosterone treatment. The other half of the clutch received a control treatment (0.1 ml of ethanol per litre of tank water). After 24 h of treatment, the eggs were collected and rinsed with clean water. Half of the testosterone-treated and control eggs were frozen for hormone analyses and the other half was left to hatch.

Rotational Preference Test with Predator

This test was based on the test developed by Bisazza et al. (1997) and was mostly identical to the procedure of the rotational preference test in the study of Schaafsma & Groothuis (2011). In short, it consisted of a circular tank (diameter 48 cm) of which the inside was covered by black polypropylene (Vikuprop, Vink Kunststoffen BV, Didam, The Netherlands). In the middle of the tank a smaller transparent circular tank was placed (diameter 20 cm) which contained the predator (of which four individuals were used in total; standard length mean ± SD = 13.02 ± 0.62 cm) restrained from moving by means of two Plexiglas plates. The tank was filled with 15 cm of water and lit from above with a 60 W light bulb. Two of these identical devices were placed in a completely darkened room. The subject fish were allowed to acclimatize in a small opaque PVC tube (diameter 5 cm), which was positioned anterior to the predator for 5 min. Thereafter, the light was switched on, the tube was gently removed, and behaviour recorded for 15 min. The recordings were analysed using the software program Observer 6.0 (Noldus Information Technology, Wageningen, The Netherlands). Time spent swimming in a clockwise or anticlockwise position was computed. We excluded from analysis the time fish spent turning to change their viewing position or trying to escape from the apparatus (facing the outer wall).

Rotational Preference Test with Mirror

This test consisted of a circular tank (diameter 30 cm) with mirror foil attached to the inner wall. The tank was filled to the edge of the mirror foil with water (15 cm). The subject fish was gently dip-netted into a small opaque PVC tube (diameter 5 cm) placed in the middle of the apparatus and left to acclimatize for 5 min in total darkness. Then the light was turned on, the tube gently removed, and behaviour recorded for 15 min. The recordings were analysed in the same way as described for the RPT-P.

Sex Determination

Immediately after the behavioural tests the fish were placed in a 200 ml opaque cup containing a clove oil and water mixture that had been thoroughly mixed, a technique commonly used to euthanize fish (see Ethical note below). After euthanasia the fish were decapitated and immediately frozen at −80 °C. At the time of histology the euthanized fish were taken out of the freezer, the tail and epaxial musculature were removed and transverse serial sections were cut at 12 μm, stained with haematoxylin-eosin Y and examined under a light microscope. We scored the presence of ovarian or testicular tissue. Hermaphrodite fish were not found. Sex determination revealed 22 males (12 controls) and 44 females (19 controls). In 26 fish no ovarian or testicular tissue was found.

Determination of Egg Hormone Levels

Egg testosterone levels were quantified by radioimmunoassay after extraction. First, hormones were extracted from egg samples using a modification of a protocol used previously on salmonid eggs (Eriksen et al. 2006). Briefly, samples were weighed (0.001 g), and crushed in a glass Potter homogenizer after adding 500 μl of 100% methanol. The sample was transferred to a glass tube, and the Potter tube and rod were each rinsed twice with 500 μl of 100% methanol and then added to the glass tube. The sample was then dried under nitrogen at 50 °C, and extracted in 3 ml of 70% diethyl ether (DEE) and 30% petroleum benzine (PB), vortexed for 60 s and centrifuged (2000 rpm, 5 min, 4 °C). This process was repeated twice (addition of 3 ml 70% DEE/30% PB, 30 s and 15 s vortex, respectively). All extracts from each sample were combined and dried under nitrogen at 37 °C. Next, 2 ml of 70% methanol was added and the sample was vortexed and left for 3 days at −20 °C. Then, the extracts were centrifuged for 5 min (2000 rpm, 4 °C), decanted and dried under nitrogen at 50 °C. Subsequently, extracts were dissolved in 150 μl of phosphate-buffered saline with gelatine. From this solution, a subsample of 20 μl was taken, mixed with scintillation cocktail (Ultima Gold, Perkin Elmer) and radioactivity (^{3}H, Perkin Elmer) counted on a liquid scintillation counter. Standards for each assay were prepared using dilution series from preprepared stock and ranged from 0.04 to 20 ng/ml of testosterone. Egg dilution curves ran parallel to the standards. Intra-assay CV was 3%.

Statistical Analysis

Five of the 22 males (two testosterone) and five of the 44 females (all testosterone) were immobile during the RPT-P and were removed from further analyses. In the RPT-M five males (four testosterone) and five females (four testosterone) were immobile during the test and were removed from further analyses.

Following the literature (e.g. Bisazza & Vallortigara 1997), we calculated a laterality index (LI). In the RPT-P the LI was calculated using the formula: (time spent swimming anticlockwise − time spent swimming clockwise)/(time spent swimming anticlockwise + time spent swimming clockwise). In the RPT-M the LI was calculated as: (time spent swimming clockwise − time spent swimming anticlockwise)/(time spent swimming clockwise + time spent swimming anticlockwise). In this manner, the formulas calculating the LIs in the RPT-P and RPT-M both resulted in positive

values when the left eye was used more, and negative values when the right eye was used more, because in the RPT-P test anticlockwise swimming indicates fixating the predator with the left eye while in the RPT-M test anticlockwise swimming indicates viewing itself in the mirror with the right eye.

Also, the absolute value of the laterality index was calculated to investigate the strength of lateralization, independent of the direction.

After we applied an arcsine square-root transformation, the LIs followed a normal distribution. The log $(x \times 100 + 1)$ transformations of the absolute values of the LI were not completely successful in normalizing the data, but the residuals of the models (in contrast to the residuals of the models when the untransformed variable was used) followed a normal distribution. Variances of the dependent variables were not significantly different across groups in all models. The LI and |LI| were analysed using hierarchical models in the software program MLwiN 2.02 (Rasbash et al. 2005). We created two levels, the highest being clutch ID and the lowest fish ID. Treatment and sex were the categorical predictive factors. The models were estimated using restricted iterative generalized least squares. Significance of the fixed factors was tested using the Wald statistic, which follows a chi-square distribution.

To test whether groups showed a population bias of lateralization, we conducted one-sample *t* tests. To investigate whether our hormone treatment was effective we used a Wilcoxon signed-ranks test, as hormone concentrations concerned paired, non-normally distributed data. These statistical analyses were performed in the software program SPSS 16.0.2 (SPSS Inc., Chicago, IL, U.S.A.). All tests were two tailed and significance level was set at $\alpha < 0.05$.

Ethical Note

Hatching success and survival of the larvae over the first few days after hatching was low in four of eight clutches, irrespective of treatment. As clutches were not attended by their parents, which would normally fan and clean the eggs, this lack of parental care might have been the primary cause. We had no indications that the welfare of fish that were exposed to pT levels elevated within their physiological range was impaired. No adverse effects of testosterone were seen on survival or growth. Fish were killed by an overdose of clove oil (1 g/litre). We used clove oil in more than five times the anaesthetic solution (maximum 100 mg/litre for induction in fish, Neiffer & Stamper 2009). Clove oil has been shown to be an appropriate anaesthetic in fish, inducing anaesthesia quicker than MS-222 (Perdikaris et al. 2010) and is a recommended method for euthanasia in fish (ANZCCART 2001). Opercular movement ceased within 1 min and fish were left in the solution additionally for at least 10 min. After euthanasia the fish were decapitated and immediately frozen at −80 °C.

The predators, being hand tame and very calm in our tanks, were restrained during the behavioural trials by means of two Plexiglas plates to prevent any lateralized interactions between the predator and the subject fish. These plates were loosely placed in such a manner that the fish could still move but could not turn. The predators showed tonic immobility when first placed in this position, but after approximately 2 min showed a normal posture and no changes to a darker colour (a sign of stress in many cichlid fish) were noted. Individual *A. ocellatus* were used for a maximum of 1 h and reused after a 47 h period of recovery in the home tank. After the experiment they were returned to their home tanks and were kept for possible reuse. All experimental protocols were approved by the ethical committee for animal research of the University of Groningen.

RESULTS

Treatment of Eggs

The testosterone treatment significantly affected the concentration of testosterone in the eggs after 24 h (control: median = 1.15 pg/mg; testosterone: median = 3.13 pg/mg; Wilcoxon signed-ranks test: $Z = -1.960$, $N = 8$, $P < 0.05$). The testosterone-treated eggs showed an increase in testosterone 2.76 times the standard deviation of the control eggs (SD = 0.717).

LI during Rotational Test with Predator

No sex effect in LI was apparent in the control fish ($\chi^2_1 = 0.149$, $P = 0.699$). Although the control fish viewed the predator as expected mainly with their right eye (Fig. 1a), this population bias was not significant (one-sample $t_{37} = 0.152$, $P = 0.880$). However, we did find a sex-specific treatment effect. Control females viewed the predator mainly with their right eye whereas testosterone-treated females looked at the predator mainly with their left eye resulting

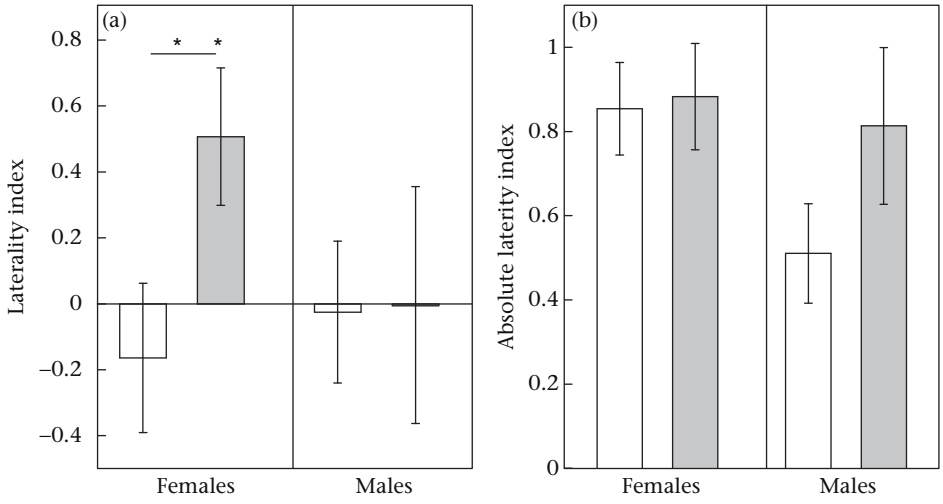

Figure 1. (a) Laterality index (arcsine square root) and (b) absolute laterality index (log) of the rotational preference test with a predator as stimulus (RPT-P) for females ($N_{control} = 19$, $N_{testosterone} = 20$) and males ($N_{control} = 9$, $N_{testosterone} = 8$) in the control (white bars) and testosterone (grey bars) treatment groups. Positive values of the LI indicate more left-eye use. Means and SEMs are shown. $^*P < 0.05$.

in a significant treatment effect ($\chi^2_1 = 4.764$, $P = 0.029$). Males did not react to the treatment ($\chi^2_1 = 0.01$, $P = 0.975$) and did not show a significant preference for either eye to view the predator (Fig. 1a). Nevertheless, there was no significant interaction effect between treatment and sex present on LI ($\chi^2_3 = 5.630$, $P = 0.131$), possibly because of the low sample size. Post hoc tests show that testosterone-treated females showed a significant population bias to look at the predator with their left eye (one-sample $t_{19} = 2.433$, $P = 0.025$,) whereas none of the other groups showed a significant population bias (all P values > 0.477; Fig. 1a).

Control females showed a nonsignificant trend to be more strongly lateralized than control males ($\chi^2_1 = 3.148$, $P = 0.076$; Fig. 1b). Treatment had no effect on the absolute LI in females ($\chi^2_1 = 0.117$, $P = 0.732$) or in males ($\chi^2_1 = 1.692$, $P = 0.193$); neither was there an interaction effect between treatment and sex ($\chi^2_3 = 2.692$, $P = 0.442$; Fig. 1b).

LI during Rotational Test with Mirror

No sex effect in LI was apparent in the control fish ($\chi^2_1 = 0.001$, $P = 0.975$). As expected, the control fish showed a significant population bias to view their mirror image with the left eye (one-sample $t_{40} = 2.260$, $P = 0.029$). The testosterone-treated fish did not show population bias lateralization in this test (one-sample $t_{39} = 1.308$, $P = 0.198$). No effect of treatment was detected on the LI in either sex (females: $\chi^2_1 = 0.013$, $P = 0.909$; males: $\chi^2_1 = 1.634$, $P = 0.201$; treatment * sex: $\chi^2_3 = 1.136$, $P = 0.768$; Fig. 2a).

No sex effect was apparent in the absolute value of the LI in the control fish ($\chi^2_1 = 0.034$, $P = 0.853$). There was no effect of the treatment on the females ($\chi^2_1 = 0.347$, $P = 0.556$) or on males ($\chi^2_1 = 1.125$, $P = 0.289$), and no interaction effect between treatment and sex ($\chi^2_3 = 2.761$, $P = 0.430$) on the absolute value of the LI (Fig. 2b).

DISCUSSION

In this study we aimed to test the influence of maternal androgen variation in eggs on offspring lateralization by means of artificial elevation of egg testosterone concentrations within the physiological range in a cichlid fish. Results of the hormone assay showed that our treatment was successful in elevating yolk hormone levels in the upper physiological range of this species. We performed two lateralization tests of visually guided behaviour, and expected, based on the literature, that the control fish would view a predator mainly with the right eye (Bisazza & Vallortigara 1997; Bisazza et al. 1999; Facchin et al. 1999; Brown et al. 2004) and their mirror image with their left eye (Sovrano et al. 1999, 2001; de Santi et al. 2001; Sovrano & Andrew 2006). Although some contradictory results have also been reported concerning the latter test (right eye use for mirror viewing: Bisazza & de Santi 2003; Reddon & Balshine 2010; Arnott et al. 2011), two of these studies obviously concerned lateralization of aggression and one (Reddon & Balshine 2010) used adult cichlid fish known to fight their mirror image (Riebli et al. 2011). Lateralization of aggression seems to be different from lateralization of viewing a conspecific, which is probably related to schooling. We analysed the results in a sex-specific way. The results of the tests show that the population bias in the control group was in the most commonly observed direction both while viewing a predator and while viewing their mirror image outside an agonistic context, but was only significant for the latter test. However, even in this mirror test the effect was subtle such that individual fish could show no bias or even the opposite bias of that at the population level.

Our results show that pT changed the direction of lateralization while viewing a predator in female, but not in male *A. rivulatus*. Control females showed a nonsignificant bias to view the predator with the right eye, whereas females that were exposed to increased levels of testosterone prenatally showed a significant population bias to view the predator with the left eye. Such a shift in the direction of lateralization in females only is in agreement with a study in Wistar rats, although lateralization in the rat study concerned motor behaviour not visually guided behaviour (Rosen et al. 1983). In contrast to our finding, Schwarz & Rogers (1992) found that in chickens testosterone treatment administered on day 16 of incubation reversed the pattern of asymmetry in the brain of males whereas it decreased lateralization in females. However, these authors investigated part of the visual pathway by examining the brain anatomy of very young chicks (6 days posthatching) and not the behaviour in almost mature animals as we did.

The current study does not support the sexual differentiation hypothesis (Hines & Shipley 1984) as females did not shift in

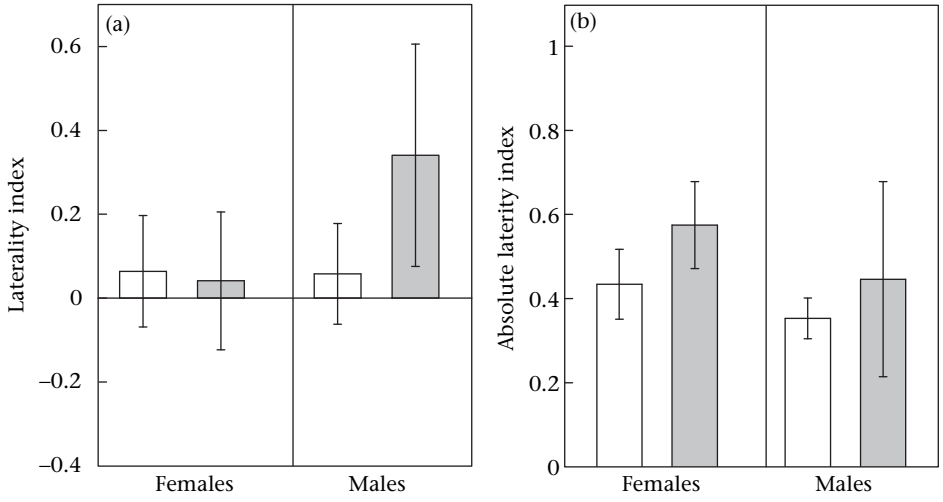

Figure 2. (a) Laterality index (arcsine square root) and (b) absolute laterality index (log) of the rotational preference test with the Fish's mirror image as stimulus (RPT-M) for females ($N_{control} = 18$, $N_{testosterone} = 21$) and males ($N_{control} = 11$, $N_{testosterone} = 6$) in the control (white bars) and testosterone (grey bars) treatment groups. Positive values of the LI indicate more left-eye use. Means and SEMs are shown. *$P < 0.05$.

direction of lateralization towards a more masculine pattern when the fish were viewing the mirror image and the sex difference in lateralization in control fish was not significant. Our results partly support the Geschwind & Galaburda (1985) hypothesis, as we found an increase in right-hemisphere dominance in fish that were prenatally exposed to testosterone, in the predator viewing task, but this was only true for females. Moreover, this hypothesis is based on sex differences in humans, whereas we did not find such differences in control fish. Furthermore, in contrast to the prediction, no significant increase in strength of lateralization was observed when viewing the mirror image.

We did not find an effect of pT on lateralization in males, but we did in females, suggesting that the latter are more sensitive to pT exposure, or that prenatal or perinatal testosterone production by males is higher than by females and overrides the effect of maternal testosterone. In any case, our results suggest that mothers may influence the direction of lateralization of daughters but not sons. In this species, however, sons are sensitive to postnatal testosterone, which affects the direction of lateralization in males and not in females (Schaafsma & Groothuis 2011), perhaps acting as a means for males to adjust their direction of lateralization.

Strength of lateralization was not affected by hormone treatment. Although in another fish species direction was shown to have a heritable component (Bisazza et al. 2001), in cichlid fish, strength, but not direction, of lateralization is a heritable trait (Brown et al. 2007). Possibly, strength of lateralization is a genetic trait, whereas direction of lateralization is under the influence of (heritable) environmental factors such as gonadal hormones.

Prenatal testosterone did not have an effect on the lateralization pattern during the rotational preference test with their mirror image as stimulus. The finding that pT affects one behavioural domain of lateralization but not another is in agreement with the human literature in which pT correlated differently with lateralization patterns of language and handedness (Lust et al. 2011).

Both predator–prey interactions and agonistic and synergetic interactions within species can theoretically lead to population-level lateralization (Ghirlanda & Vallortigara 2004; Ghirlanda et al. 2009, respectively). However, population-level lateralization in tasks that measure behaviours related to these different evolutionarily stable strategies does not have to be cross-correlated (Sovrano et al. 1999). Predator pressure can be highly variable in different habitats for the same species (e.g. Brown 2004; Bell & Sih 2007; Dingemanse et al. 2007), whereas conspecifics will always be around; thus different costs and benefits have been suggested to be associated with population-level lateralization with respect to antipredator and social behaviours. Strong population lateralization in species regarding predatory behaviour could be exploited by the predator, whereas this disadvantage may be smaller for interspecific interactions, as it may promote coordination of schooling behaviour (Bisazza & Dadda 2005) and minimize fighting costs (Arnott et al. 2011). Therefore, more environmentally induced flexibility in the direction of lateralization for viewing predators than conspecifics may be adaptive.

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